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## Introduction.

It has been increasingly recognized that cancer cells actively recruit stromal cells into the tumors and that this recruitment is essential for the generation of a microenvironment that promote tumor growth<sup>1-9</sup>. The presence of a large number of myofibroblasts is apparent in the stromal compartment of most invasive human breast cancers but not found in the stroma of normal breast tissue<sup>10</sup>. Myofibroblasts are stromal fibroblasts with features of both myoblasts (e.g., expression of smooth muscle actin) and fibroblasts that have been implicated in breast cancer invasion, extracellular matrix remodeling, wound healing, and chronic inflammation<sup>11-13</sup>. The cell type of origin of myofibroblasts is not conclusively established. Isolation of various stromal and epithelial cells from breast tumors and their co culturing in vitro demonstrated that cancer epithelial cells can induce the expression of myofibroblast markers in a subset of fibroblasts<sup>14</sup>. However, the finding that only a small fraction of fibroblasts were transformed into myofibroblasts<sup>14</sup> raises the question of whether myofibroblasts could be derived from specific stem cells that are recruited by cancer epithelial cells from either bone marrow or locally in the adjacent breast adipose tissue. Recent data both in animal models and human breast tumors support the hypothesis that at least a subset of cancer-associated myofibroblasts is derived from circulating bone-marrow derived cells<sup>15-17</sup>. However, the involvement of adipose tissue derived stem cells (ASCs) in tumor growth has not been reported. We recently showed that ASCs give rise to tumor growth of highly vascularized tumors when co-injected with 4T1 cancer cells<sup>18</sup>.

The precise cellular and molecular mechanisms that dictate metastasis of a specific tumor to a predetermined metastatic location are not known. Many tumors have a predilection for metastasis to specific organs. Based on the current dogma, metastatic predisposition is believed to reflect inherent molecular differences in tumor cells themselves and the potential influence by surrounding stromal cells, which include the vasculature, connective tissue and immune cells<sup>19-23</sup>. Kaplan et al demonstrated that tumor metastasis is initiated by a well-defined sequence of events dependent on cellular 'bookmarking' through site-specific delivery of VEGFR1<sup>+</sup>/c-kit<sup>+</sup> cells to form permissive niches within target organs. Furthermore, they showed that differences in tumor-secreted humoral factors promote metastatic spread in specific distant organs. Within days following tumor implantation, fibronectin becomes up regulated in certain locations by resident fibroblast and fibroblast-like cells within target organs that are conventional sites of metastasis, corresponding to the particular primary tumor. As a result of the niche-specific directional cues from fibronectin, VEGFR1<sup>+</sup>/c-kit<sup>+</sup> cells, expressing VLA-4, can traverse established endothelium to form a pre-metastatic niche before the arrival of CXCR4<sup>+</sup> tumor cells and VEGFR2<sup>+</sup> endothelial cells. These clusters, with MMP9 production altering the microenvironment and enhanced expression of SDF-1 creating a chemokine gradient, permit the attraction of tumor cells and their incorporation into the niche, thereby developing a complete metastatic lesion.

The objective is to study the role of adipose tissue derived stem cells (ASCs) in breast cancer growth and metastasis.

## **Body.**

### ***Methods:***

#### *Isolation of mASC.*

Perirenal, pelvic and subcutaneous fat tissue were dissected from EGFP-transgenic mice, washed in PBS and immediately processed. After mincing the tissue in pieces  $< 2\text{mm}^3$ , serum-free  $\alpha$ -MEM (1ml/1g tissue) and 2 units/g tissue Liberase Blendzyme 3 (Roche) was added and incubated under continuously shaking at  $37^\circ\text{C}$  for 45 min. The digested tissue was sequentially filtered through 100- $\mu\text{m}$  and 40- $\mu\text{m}$  filters (Fisher Scientific) and centrifuged at 450 g for 10 min. The supernatant containing adipocytes and debris was discarded, and the pelleted cells were washed twice with Hanks' balanced salt solution (Cellgro) and finally resuspended in growth media. Growth media contained  $\alpha$ -modification of Eagle's medium (Cellgro), 20% FBS (Atlanta Biologicals), 2 mM glutamine (Cellgro), 100 U/ml penicillin with 100  $\mu\text{g/ml}$  streptomycin (Cellgro). Plastic adherent cells were then grown in Nunclon culture vials (Nunc) at  $37^\circ\text{C}$  in a humidified atmosphere containing 5%  $\text{CO}_2$  followed by daily washes to remove red blood cells and nonattached cells. After 80% confluence of passage 0, cells were seeded at a density of 3,000 cells/ $\text{cm}^2$  (passage 1).

#### *Cell lines and cell culture techniques.*

Breast cancer cell lines 4T1 MDA-MB-231, MCF7 and MDA-MB-435 cells were purchased from ATCC and cultured in RPMI 1640, Leibovitz's L-15 Medium, Eagle's Minimum Essential Medium, and Leibovitz's L-15 Medium, medium, respectively, supplemented with 10% heat-inactivated Fetal Bovine Serum (Atlanta Biologicals Inc), 2 mM Glutamine, 100 units/ml Penicillin and 100  $\mu\text{g/ml}$  Streptomycin.

#### *Animal experiments.*

The following experiments were performed to investigate whether adipose tissue derived stem cells form pre-metastatic niche prior to the arrival of tumor cells. The recipient mice (wild-type BALB/c mice) were lethally irradiated (950 rads) and transplanted with  $1 \times 10^6$  GFP<sup>+</sup> ASCs from EGFP-transgenic mice (C57Bl/6-TgN(ActbEGFP)10sb/J; Jackson Laboratory). The animals were then divided into 5 groups (n=10/group) receiving either tumor cell injection or PBS. Tumor cells were tagged with DsRED. Group 1: 4T1, group 2: MDA-MB-231; group 3: MCF7; group 4: MDA-MB-435; group 5: PBS. Fourteen and 18 days after tumor cell implantation, mice were killed and lungs collected for further analysis. For visualization of GFP+stem cells and DsRED+ tumor cells, tissues were immediately frozen in OCT compound (Tissue-Tek) without fixation. Serial sections were mounted with Vectashield containing DAPI (4,6-diamidino-2-phenylindole), and visualized with a fluorescent microscope. For immunohistochemistry (anti c-kit, anti-VEGFR1 and VEGFR2 from Abcam), lungs were fixed and embedded in OCT. To further define the timing of tumor cell arrival, a flow cytometric study of the lungs was undertaken. Lungs were collected after perfusion with PBS by right-ventricular injection. The tissues were minced into small pieces, filtered with 100- and 40- $\mu\text{m}$  filters (BD Biosciences) to form a single-cell suspension.

**Result:**

After irradiation, but before tumor implantation, we observed no GFP<sup>+</sup> ASCs in the lungs (Fig 1A). By day 14 after 4T1 tumor implantation, but before the arrival of tumour cells, the extravasation and cluster formation of GFP<sup>+</sup> ASCs were detected in the lungs (Fig. 1B). Individual DsRed-tagged tumour cells, associated with pre-existing ASC clusters, were visible by day 18 (Fig. 1C). To further define the timing of tumour cell arrival, a flow cytometric study of the lungs was undertaken. Before day 5, minimal GFP<sup>+</sup> ASCs were observed in this tissue; however, from day 10, ASCs began migrating into the lung (Fig. 1D). These GFP<sup>+</sup> cells increased in number, and were joined by DsRed-tagged tumor cells by day 18. No tumor cells were detected by flow cytometry or microscopy earlier than day 18. Studies from MDA-MB-231, MCF7/Ras and MDA-MB-435 showed similar results.

In order to determine the functional role for VEGFR1<sup>+</sup> ASC in directing metastasis, we assessed the potential of purified VEGFR1<sup>+</sup> ASC cells to initiate pre-metastatic clusters by selectively transplanting these progenitors into irradiated mice (n=10/group). Group 1: VEGFR1<sup>+</sup> ASC cells; group 2: VEGFR1 depleted ASC cells. By day 24 after tumor cell implantation, we observed that mice transplanted with purified VEGFR1<sup>+</sup> cells formed numerous micrometastases throughout the lungs (Fig. 2). In contrast, we observed that VEGFR1 depleted cells failed to produce pre-metastatic clusters ( $P < 0.01$  by analysis of variance, ANOVA). These results suggest that the VEGFR1<sup>+</sup> ASCs initiated the pre-metastatic cluster which can attract tumor cells.

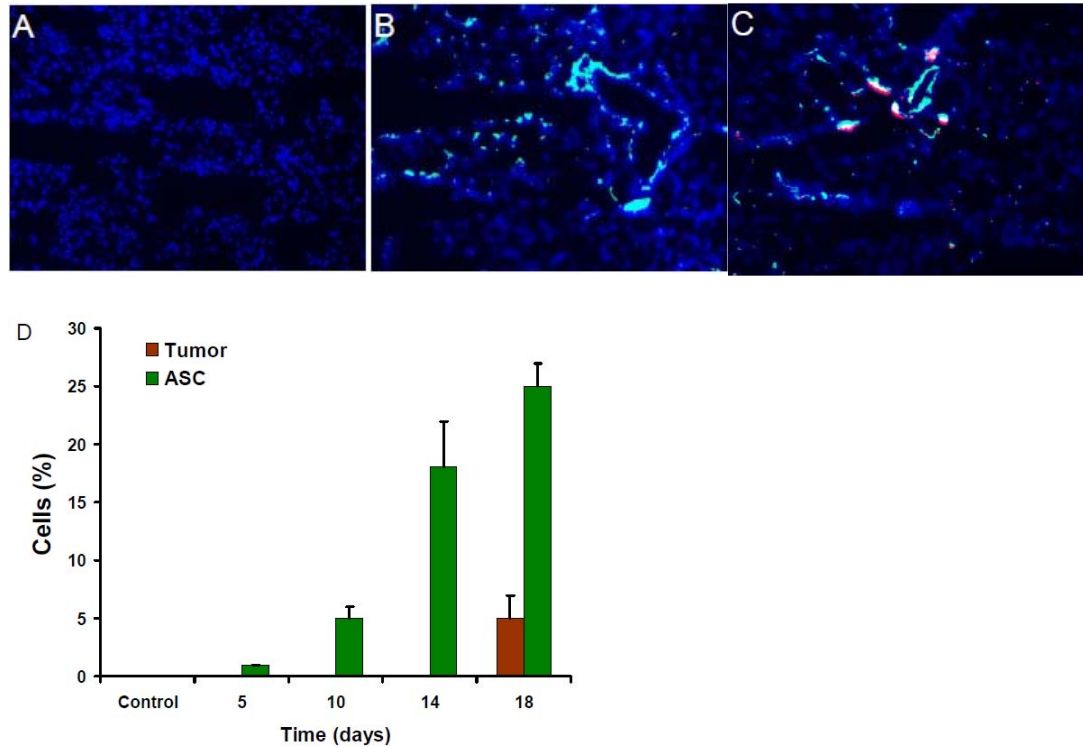


Figure 1. Adipose tissue derived stem cells form the premetastatic niche. **A**, GFP<sup>+</sup> ASCs are not found in the lungs after irradiation and before DsRed-tagged 4T1 cell implantation ( $n = 5$ ). **B**, On day 14, GFP<sup>+</sup> (green) ASCs are seen with no DsRed<sup>+</sup> (red) tumour cells ( $n = 5$ ). **C**, Beginning on day 18, a few single DsRed<sup>+</sup> 4T1 cancer cells adhere to GFP<sup>+</sup> ASC clusters ( $n=5$ ). **D**, A graph showing flow cytometric data of GFP<sup>+</sup> ASCs and DsRed<sup>+</sup> 4T1 cancer cells in the lung.

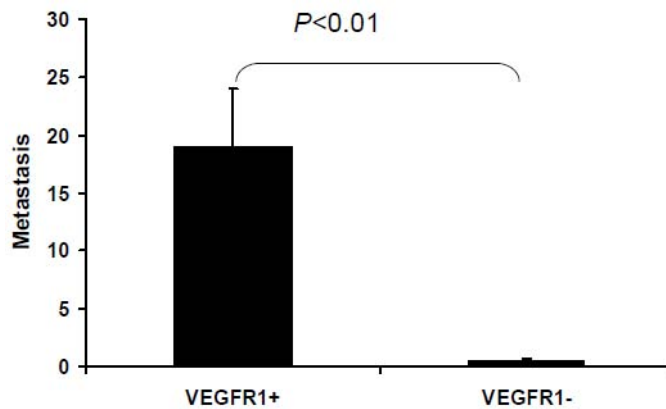


Figure 2. VEGFR1<sup>+</sup>-selected ASCs permits micrometastasis. VEGFR1<sup>+</sup> and VEGFR<sup>-</sup> cells were separated by cell sorting. Purity of VEGFR1<sup>+</sup> cells was 95%. The purified cells were injected intravenously ( $10^5$  cells) every three days for a total of 23 days. Animals were sacrificed 24 days after 4T1 tumor cell implantation. ASCs depleted of VEGFR1<sup>+</sup> cells abrogates both clusters and metastases (right panel) ( $P < 0.01$  by ANOVA). The graph shows the number of 4T1 micrometastases per x100 objective field.

### **Key Research Accomplishments**

- The project on the bone marrow transplantation, reconstitution and formation of adipose tissue derived stem cells (ASC) derived clusters in the lung has been accomplished in time as originally planned in the Statement of Work of the grant proposal.
- We detected GFP<sup>+</sup> cells in the lung prior to the arrival of tumor cells and the co-localization of both GFP<sup>+</sup> and tumor cells was detected at later time points. These data indicate that ASCs formed a niche in preparation for the arrival of tumor cells. These encouraging data confirmed our hypothesis that breast tumor cells produce growth factors/cytokines that direct the mobilization and homing of a specific subpopulation ASCs to pre-metastatic sites to form a niche before the arrival of tumor cells.
- Purified VEGFR1<sup>+</sup> cells but not VEGFR1<sup>-</sup> cells are responsible for to initiate pre-metastatic clusters. These data are important because it forms the foundation for the continuation of the remaining proposed projects to determine the cellular and molecular mechanisms by which migratory stem cells, through interaction with the microenvironment, form permissive pre-metastatic niches and to define specific growth factors/cytokines responsible for the mobilization and homing of ASCs to pre-metastatic sites.



### **Reportable Outcomes**

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Tissue resident stem cells produce CCL5 under the influence of cancer cells and thereby promote breast cancer cell invasion. ***Cancer Lett.*** 2009 May 6. [Epub ahead of print].

## **Conclusion**

The project on the bone marrow transplantation, reconstitution and formation of adipose tissue derived stem cells (ASC) derived clusters in the lung has been accomplished in time as originally planned in the Statement of Work of my grant proposal. We detected GFP<sup>+</sup> cells in the lung prior to the arrival of tumor cells and the co-localization of both GFP<sup>+</sup> and tumor cells was detected at later time points. These data indicate that ASCs formed a niche in preparation for the arrival of tumor cells. These encouraging data confirmed our hypothesis that breast tumor cells produce growth factors/cytokines that direct the mobilization and homing of a specific subpopulation ASCs to pre-metastatic sites to form a niche before the arrival of tumor cells. These data are important because it forms the foundation for the continuation of the remaining proposed projects to determine the cellular and molecular mechanisms by which migratory stem cells, through interaction with the microenvironment, form permissive pre-metastatic niches and to define specific growth factors/cytokines responsible for the mobilization and homing of ASCs to pre-metastatic sites. We believe that all of the remaining proposed studies will be accomplished within the time frame that was originally planned.

## **So What Section.**

It is known that stromal cells contribute to the development of a wide variety of tumors. There is a higher incidence of tumor formation in tissues exhibiting a chronically inflamed stroma as well as those undergoing various types of wound healing, in which the stroma plays a central role. Stromal cell compartments contain a variety of mesenchymal cells such as fibroblasts, myofibroblasts, endothelial cells, pericytes, and a variety of inflammatory cells associated with the immune system. However, the involvement of adipose tissue derived stem cells in breast cancer stem cell niche formation at metastatic sites has never been investigated. Our findings shed new light to the mechanisms of tumor metastasis and will pave the way to new therapies targeting the stem cell niches which is necessary for tumor metastasis.

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# Tissue resident stem cells produce CCL5 under the influence of cancer cells and thereby promote breast cancer cell invasion

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## ABSTRACT

In the present study, we investigated whether human adipose tissue derived stem cells (hASCs) could enhance tumor invasion and whether these hASCs could be a potential source of CCL5. We observed a significant increase in the number of breast cancer cells that invaded the matrigel when Co-cultured with hASCs. We found that hASCs produce CCL5 in the Co-culture and cancer cell invasion was diminished by an antibody against CCL5. Furthermore, cancer cell invasion in the Co-culture was associated with an elevated level of MMP-9 activity. We conclude that CCL5 plays a crucial role for tumor invasion in the interplay of tissue resident stem cells from the fat tissue and breast cancer cells.

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## 1. Introduction

Stromal cells contribute to the development of a wide variety of tumors. There is a higher incidence of tumor formation in tissues exhibiting a chronically inflamed stroma as well as those undergoing various types of wound healing, in which the stroma plays a central role [1,2]. Stromal cell compartments contain a variety of cell types such as fibroblasts, myofibroblasts, endothelial cells, pericytes as well as inflammatory cells. Tumor stromal cells produce CCL5 – a chemokine which is involved in tumor progression [3–6]. Recent studies demonstrated that bone marrow derived stem cells (BMSCs) are involved in tumor stroma formation [7,8]. Furthermore BMSCs produce CCL5 when in direct Co-culture with breast cancer cells. However, the involvement of tissue resident stem cells in tumor stro-

ma formation has not been investigated so far. We and others have shown that adipose tissues contain multipotent stem cells which secrete various paracrine factors. Since breast tissue contains large amounts of adipose tissue, we sought to investigate the potential contribution of hASCs to breast cancer invasion.

## 2. Materials and methods

### 2.1. Isolation and culture of cells

Human adipose tissue was obtained from elective body contouring procedures in compliance with the guidelines of the M.D. Anderson Cancer Center Institutional Review Board. Tissue was minced by sharp dissection. Minced specimens were added to a solution of 0.07% blendzyme 3 (F. Hoffman-La Roche Ltd., Basel, Switzerland), digested with mild agitation at 37 °C for 60 min, passed through a 40 µm filter and finally selected based on adherence to T75 tissue culture flasks at 24 h. Cells were grown in alpha MEM medium supplemented with 20% fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml

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streptomycin. Cells were incubated in a 5% CO<sub>2</sub>-containing chamber at 37 °C with medium changed every 3 days. hASCs between Passages 1 and 8 were used for all experiments. hASCs used in these experiments have previously been characterized by Bay et al. group [9].

MDA MB 231 (American Type Culture Collection) were grown in MEM (Life Technologies) supplemented with 10% FBS and penicillin–streptomycin at 37 °C in 5% CO<sub>2</sub>.

## 2.2. Lentiviral GFP transfection

Stable GFP labeling was performed with a third-generation lentivirus system as described previously by our group [10,11].

## 2.3. Cell staining

Cells that were 80% confluent in a T25 flask were incubated at 37 °C in 5% CO<sub>2</sub> with 3 ml of their regular culture-medium, containing 15 µl of DiI (or 21 µl of DiO, respectively) for 1 h. In the next step cells were washed twice with PBS, before regular medium was added for a 5 min incubation time. This step was repeated and cells were then harvested and seeded for the following experiment.

## 2.4. Proliferation assay

MDA MB 231  $\times 10^4$  cells were seeded in 6-well plates in 2 ml of MEM (10% FBS). After 24 h medium was exchanged to MEM (5% FBS) in the control group and to stem cell conditioned medium (conditioning time 48 h) in the treatment group. Cell number was counted using a hemocytometer and viability assessed with Trypan Blue exclusion method.

## 2.5. Invasion assays

The chemoinvasion assay was performed using a Boyden chamber with filter inserts (pore size, 8 µm) coated with Matrigel in 24-well dishes (BD Biosciences Bedford, MA) as described previously [12]. Before performing the invasion assay, cells were mixed together in 5% FBS containing MEM to be seeded in direct Co-culture.  $35 \times 10^3$  cells (MDA MB 231),  $70 \times 10^3$  cells (hASCs/WI-38/HMECs) or in direct Co-culture (ratio of 1:2, MDA MB 231:hASCs), respectively were placed in 600 µl of 5% FBS containing MEM in the upper chamber, and 750 µl of the same medium containing 10% FBS was placed in the lower chamber. The plates were incubated for 48 h at 37 °C in 5% CO<sub>2</sub>. Cells on the upper side of the filters were removed with cotton-tipped swabs, and the filters were washed with PBS. Cells on the underside of the filters were examined and counted under a microscope. Green fluorescent cell signal of GFP labeled MDA MB 231 cells was counted in five randomly chosen view fields at a 10 $\times$  magnification of every insert. Each experiment was repeated at least three times.

## 2.6. ELISA

The amount of CCL5, secreted by MDA MB 231 and hASCs was measured using Quantakine ELISA kit (R&D Systems). Secreted cytokines were standardized to the

amount of cells counted in each well and expressed as pg cytokine per ml.

## 2.7. Exposure of hASCs and MDA MB 231 to conditioned medium (CM)

MDA MB 231 alone and hASCs (Passages 2–4) alone were grown in MEM + 5% heat-inactivated FBS culture medium and conditioned medium from these both cell types was harvested after 48 h and centrifuged at 1500 rpm for 5 min and supernatant was passed through Millipore sterile 50 ml filtration system with 0.45-µm polyvinylidene difluoride membrane. CM was stored at –20 °C until hASCs or MDA MB 231 cells were exposed to CM.

## 2.8. Anti-CCL5 treatment

GFP labeled MDA MB 231 co-seeded with hASCs was incubated with neutralizing CCL5-antibody (3 µg/ml) in the upper chamber of the invasion assays. Anti-human RANTES Antibody (Cat. Number: AF-278-NA) and the control Normal Goat IgG (Cat. Number: Ab-108-C) was purchased from R&D Systems, Minneapolis.

## 2.9. Zymography

MMP-9 activity was determined using a 10% zymogram (gelatin) pre-cats gel (Invitrogen Cat Number: EC6175). Preparation of cell lines was conducted by seeding the appropriate amount of cells (50,000) into each respective well of a 6-well plate. Cells were cultured at 37 °C (5% CO<sub>2</sub> atmosphere) until 80% confluency. Upon confluence media was changed to serum-free media and cells were cultured for an additional 48 h. After 48 h conditioned media was collected and mixed with equal volumes of 2 $\times$  SDS (15 µl conditioned media: 15 µl 2 $\times$  SDS). (Note: Preparation of conditioned media was not heated or reduced for detection of MMP-9 activity using zymography.) The electrical running apparatus was then prepared containing the 10% zymogram (gelatin) pre-cats gel and 1 $\times$  running buffer (12 g Tris, 57.6 g Glycine, 10% SDS, 4 L distilled water). Samples were then loaded accordingly (15 µl Invitrogen pre-stained protein ladder-cat no. 10748010) into the 10% zymogram (gelatin) pre-cat gel and allowed to run at room temperature at 125 V for 1.5 h. After electrophoresis the zymogram gel was removed and incubated (30 min with gentle agitation) at room temperature in a zymogram renaturing buffer (1:9 with deionized water). Upon incubation, the renaturing buffer was decanted and further incubated at room temperature in 1 $\times$  developing buffer (1:9 with deionized water) for 30 min. After 30 min the developing buffer was then decanted and replaced with fresh developing buffer. After equilibration with fresh developing buffer the zymogram gel was allowed to incubate overnight at 37 °C. After overnight incubation the developing buffer was decanted and replaced with Coomassie staining solution (25% MeOH, 10% acetic Acid, and 0.1% Coomassie brilliant blue-dissolved in water before adding) for 2 h at room temperature. Upon staining the zymogram gel was de-

stained (10% acetic acid, 15% MeOH) at room temperature using gentle agitation. Once destained the zymogram gel was photographed (Chemilmager 5500) to determine the areas of protease activity (clear bands).

### 3. Results

#### 3.1. Co-seeding of hASCs and tumor cells promotes invasion of cancer cells in vitro

Human breast cancer MDA MB 231 cells were seeded in different settings to determine if the number of invasive cancer cells would change given the possibility to interact with mesenchymal stem cells derived from human adipose tissue (hASCs). Therefore we established a two-dimensional direct Co-culture system of MDA MB 231 cells and hASCs. The breast cancer cells showed a regular distribution pattern and could thus easily interact with surrounding hASCs (Fig. 1a–d). In order to assess whether the proliferation state of MDA MB 231 cells could be modified by interaction with stem cells, we exposed MDA MB 231 cells to either regular growth medium or stem cell conditioned medium (SCCM) over a period of 4 days. We detected a higher number of viable cells after day four in the group treated with stem cell conditioned medium but not in the control (Fig. 1e). In separate experiments, cancer cells were either seeded alone on the surface of a matrigel coated insert or in direct Co-culture with hASCs, the human lung fibroblast cell line WI-38 or human mammary epithelial cells (HMEC), respectively. When seeding the breast cancer cells with hASCs on matrigel coated invasion membranes we found that approximately twice as many GFP-labeled cancer cells ( $105 \pm 16.97$  cells/view field) invaded the matrigel coated membrane when co-seeded with hASCs as compared to the control group ( $46 \pm 21.54$  cells/view field) in which cancer cells were seeded alone. To determine whether the observed effect was specific for hASCs, we co-seeded the cancer cells with WI-38 or HMECs. We found that neither WI-38, nor HMECs (human mammary epithelial cells immortalized with large T and SV40 antigens) has any impact on cancer cell invasion. In both control settings neither increase nor a significant decrease in the number of invasive cancer cells compared to the control group could be detected. Incubating the direct Co-culture of MDA MB 231 cells and hASCs with a CCL5 neutralizing antibody showed an inhibition of the invasion promoting effect exerted by hASCs on MDA MB 231 cells (Fig. 2).

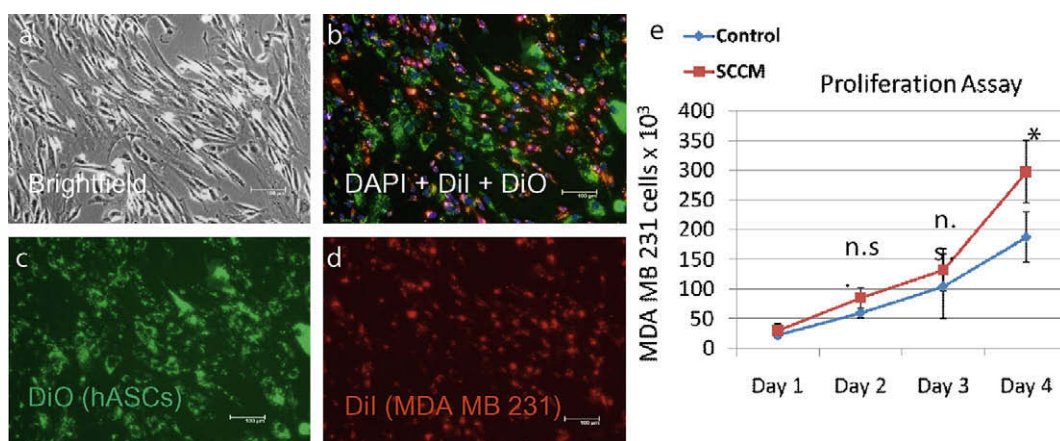
#### 3.2. Tumor derived humoral factors induce CCL5 secretion of hASCs

Previous studies have shown that breast cancer cells stimulate secretion of the chemokine CCL5 from bone marrow derived mesenchymal stem cells (BMDC), which then acts in a paracrine fashion on the cancer cells to enhance their invasion [7]. Interestingly the group of Karnoub showed that CCL5 secretion could only be induced by direct Co-culture and not by exposing BMDCs to tumor conditioned medium. In order to clarify the function of CCL5 in the interaction of breast cancer cells with tissue resident stem cells we examined the secretion of CCL5 by hASCs with ELISA.

We were not able to detect CCL5 in hASCs or MDA MB 231 cells when cultured alone (Fig. 3). However, we detected a significant amount of CCL5 in conditioned medium from hASCs and MDA MB 231 Co-cultures (Fig. 3). In order to determine the cellular source of CCL5, we added conditioned medium from MDA MB 231 cells to hASCs and found a significant amount of CCL5 produced by hASCs (Fig. 3). We also added conditioned medium from hASCs to MDA MB 231 cells and we were not able to detect CCL5 production. This data suggest that hASCs produce CCL5 under the influence of tumor cells. We furthermore measured CCL5 by using the same experimental approach to see whether the observed induction occurs also while MDA MB 231 cells are interacting with the fibroblast cell line WI-38. We did not find any secretion of CCL5 in WI-38/MDA MB 231 Co-cultures or in WI-38 cell cultures after stimulation with tumor conditioned medium (data not shown).

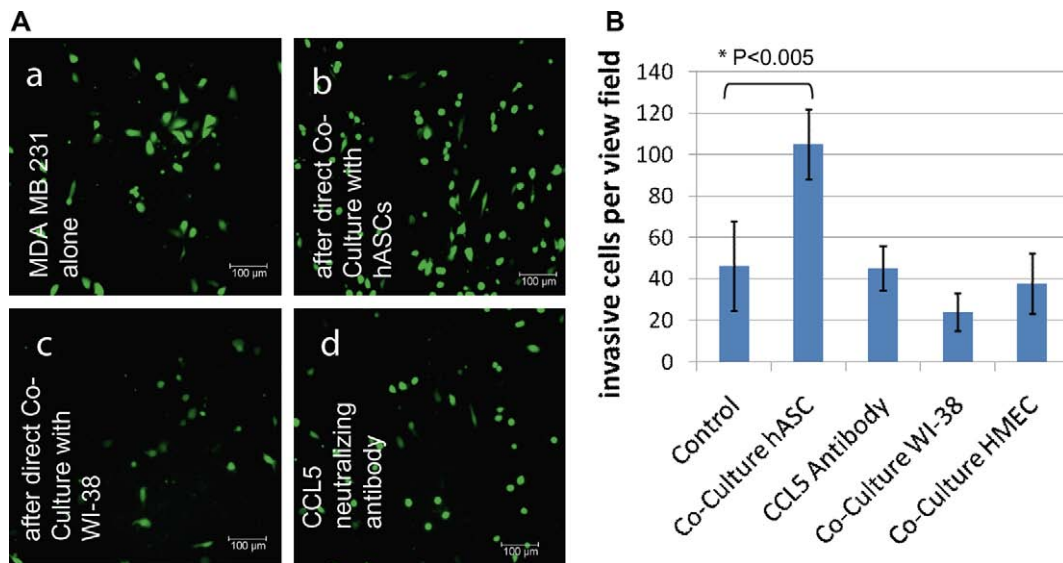
#### 3.3. CCL5 antibody blocks increased invasion of tumor cells in vitro

The importance of CCL5 secretion for cancer cell invasiveness was examined using the Boyden chamber invasion assay. A purified polyclonal neutralizing antibody against human CCL5 was added in the co-seeding invasion assays of cancer cells together with hASCs. The observed effect of hASCs on cancer cell invasion was abolished by the antibody (Fig. 2). The control IgG did not show any effect on the number of invasive cancer cells (data not shown). In order to evaluate the effect of human recombinant CCL5 on breast cancer cell invasion we exposed MDA MB 231 cells to either regular growth medium or to growth medium containing 100 pg/ml recombinant human CCL5 (Fig. 3) and found an increase in number of invasive cells ( $33.5 \pm 5$  in the CCL5 group as compared to  $14.25 \pm 7$ ) that were counted in four different view fields. The experiment was repeated three times ( $P \leq 0.005$ ).

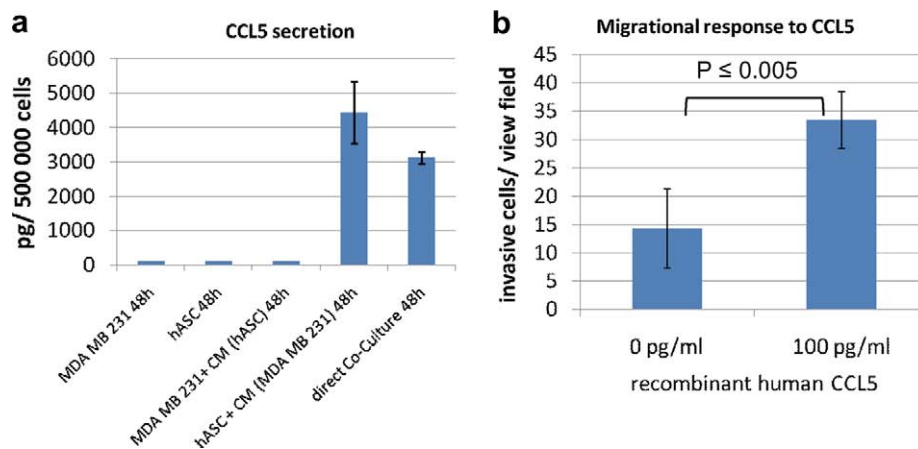


**Fig. 1.** Human adipose tissue derived stem cells and breast cancer cells in a two-dimensional direct Co-culture system. (a) Brightfield picture of direct Co-culture of MDA MB 231 and hASCs (Passage 3). (b) Overlay of DAPI (nuclei), DiI (MDA MB 231) and DiO (hASCs). (c) DiO staining of hASCs (green). (d) DiI staining of MDA MB 231 breast cancer cells (red). All images were taken in a 10 $\times$  magnification, cells were seeded in a ratio of 1:2 (MDA MB 231:hASCs). (e) Stem cell conditioned medium enhances proliferation of MDA MB 231. MDA MB 231 cells were grown in 6-well plates. The SCCM group has been exposed to stem cell conditioned medium (5% FBS, 48 h conditioning time). The control group has been exposed to regular growth medium containing 5% FBS. The SCCM group shows a higher proliferation at day 4. \* $P < 0.05$ , n.s. not significant. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)





**Fig. 2.** CCL5 is important for pro-invasive effect of hASCs on breast cancer cells. (A): (a) Green signal shows invaded GFP labeled MDA MB 231 cells when seeded alone in a density of  $35 \times 10^3$  per insert. (b) Green signal shows invaded GFP labeled MDA MB 231 cells when Co-cultured with hASCs. (c) Green signal shows invaded GFP labeled MDA MB 231 when Co-cultured with WI-38. (d) Green signal shows invaded GFP labeled MDA MB 231 when Co-cultured with hASCs in the presence of neutralizing CCL5-antibody (3  $\mu$ g/ml). The seeding ratio was always 1:2 (GFP-MDA MB 231: hASCs/WI-38). The invasion assay with a direct Co-culture of MDA MB 231 and HMEC is not represented as a picture but in the graph. (B). Quantitative data of invaded GFP-positive MDA MB 231 cells per view field is shown as mean  $\pm$  SD.



**Fig. 3.** Tumor-derived factors stimulate hASCs to secrete CCL5 and thereby promote breast cancer cell invasion. (a) MDA MB 231 cells and hASCs were either seeded alone, seeded together with MDA MB 231 or incubated with either stem cell conditioned medium CM (hASCs) or tumor conditioned medium CM (MDA MB 231) for 48 h. CCL5 level was measured by ELISA. (b) MDA MB 231 cells were either seeded alone in regular growth medium or in regular growth medium containing 100 pg/ml human recombinant CCL5 (Sigma–Aldrich, USA).

#### 3.4. Co-culture of hASCs and tumor cells increases MMP-9 activity

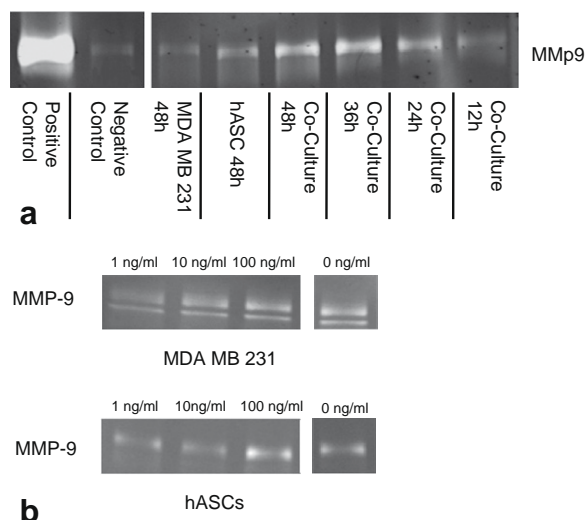
Matrixmetalloproteinases (MMPs) are enzymes that degrade the extracellular matrix and play a pivotal role in metastatic processes. It has been postulated in previous studies that CCL5 might enhance the expression of MMP-9 [13]. Therefore we examined whether MMP-9 was involved in the interplay of hASCs and cancer cells by Gel-zymography assay. After 36 h a higher activity of MMP-9 ( $86.29 \pm 11.89$  average intensity of active MMP-9 band) could be detected in the Co-culture of cancer cells and hASCs compared to the single cultures (Fig. 4a). In order to see whether CCL5 could possibly induce a higher secretion of MMP-9 we stimulated either MDA MB 231 cells or hASCs with human recombinant CCL5 in various concentrations and found that CCL5 did not increase

MMP-9 activity in MDA MB 231 cells but we found a higher average density in the group of hASCs stimulated with 100 ng/ml ( $146.147 \pm 36.882$  as compared to  $120.972 \pm 24.814$  in the control group).

#### 4. Discussion

Bone marrow derived mesenchymal stem cells have been recently found to integrate into the tumor associated stroma and secrete CCL5 which then acts in a paracrine fashion on the cancer cells to enhance their invasion [7]. However, the role of adjacent tissue resident stem cells





**Fig. 4.** MMP-9 activity in MDA MB 231 and hASCs Co-culture. MMP-9 activity was analyzed with a pre-cats (gelatin) Zymogram gel. Clear bands show MMP-9 activity. (a) MMP-9 activity was measured in at different time points in direct Co-cultures of MDA MB 231 cells and hASCs (seeding density for each sample:  $5 \times 10^4$  MDA MB 231,  $10^5$  hASCs), and in single cultures as indicated. (b) MMP-9 activity in supernatants of MDA MB 231 cells ( $5 \times 10^4$  per well) or hASCs ( $10^5$  per well, Passage 3), respectively, after stimulation with human recombinant CCL5 for 48 h, concentrations of CCL5 as indicated above each band. We used 5% FBS medium as negative control and the supernatant of 293T cells transfected with human MMP-9 cell cultures as positive control.

in breast cancer invasion has not been investigated. In this study we showed that mesenchymal stem cells derived from human adipose tissue (hASCs) promote the invasion of the human breast cancer cell line MDA MB 231 when Co-cultured together. This invasion promoting effect is specific for hASCs since the effect was not observed when MDA MB 231 cells were Co-cultured with human fibroblasts or immortalized human mammary epithelial cells. More importantly, we found that hASCs produce CCL5 when Co-cultured with MDA MB 231 cells. When exposing the hASCs and tumor cell Co-culture to a CCL5 neutralizing antibody the observed increase in number of invasive cells was blocked. Furthermore we detected an elevated level of MMP-9 activity in the Co-culture of hASCs and MDA MB 231 cells. Our results indicate that CCL5 might not only act in a paracrine fashion on MDA MB 231 cells but also affect the secretion of MMP-9 by hASCs in an autocrine fashion. To our knowledge this is the first report showing that tissue resident stem cells can be stimulated by human breast cancer cells to secrete CCL5 and thereby promote cancer cell invasion.

Several investigations have recently provided evidence for the potential contribution of the CC chemokine CCL5 to tumor progression [14,15]. In our study, CCL5 production was detected only in hASCs grown in conditioned medium from MDA MB 231 cells but not detected in MDA MB 231 cells grown in conditioned medium from hASCs. These data suggest the presence of certain factors secreted by MDA MB 231 cells that induced CCL5 expression in hASCs. Interestingly we found that CCL5 secretion could be induced by exposing hASCs to tumor conditioned

medium in contrast to the findings of Karnoub et al. [7] which showed that only direct cell contact could induce CCL5 secretion. This discrepancy may be explained by the differences between bone marrow derived stem cell and adipose tissue derived stem cells. Noel et al. [16] performed quantitative comparison between these two different cell types using Taqman Low Density Array, 2D electrophoresis and differentiation functional assays. Their data revealed cell specific differences at transcriptional and proteomic levels between both cell types according to their tissue origin as well as functional differences in their differentiation processes towards adipogenic, osteogenic and chondrogenic programs. Their observations suggest that ADSC and MSC are fundamentally different cell types and differently committed cells.

A recent study suggested that proinflammatory cytokines, IFN-gamma and TNF-alpha, promoted expression of CCL5 by breast cancer lines [14]. Another study also suggested that TNF-alpha levels were associated with increased lung concentrations of CCL5 [17]. Accordingly, we also examined the production of TNF-alpha in MDA MB 231 cells, but the level of TNF-alpha was very low (data not shown). Therefore, the factors secreted by MDA MB 231 cells that stimulate CCL5 production in hASCs remain to be identified.

One of the many tumor associated changes in the physiological structure of the affected organ is a process called desmoplasia representing the stromal response to cancer cells. Part of this structural change is the disruption of the basement membrane and a general remodeling of the extracellular matrix. The enzymes responsible for this process are mostly part of the Matrixmetalloproteinase family. MMP-9 has been shown to play a central role for the angiogenic switch and tumor invasion in several tumor models by digesting collagen and also releasing vascular growth factors sequestered in the extracellular matrix. Along this line, we found higher levels of MMP-9 activity in the conditioned medium of MDA MB 231 Co-culture. Recent reports have suggested that stromal cells are the principal source of MMP-9 in gastric cancer [18,19]. Since the hASCs reside in local adipose tissue surrounding the lobular structures of the breast they might be the most potent early response cells that initially create the tumor micro-environment. Therefore hASCs represent a cellular source providing CCL5 which influences tumor cell migration and invasion in a paracrine as well as autocrine fashion. These novel findings may pave the way to the development of a specific targeted therapy in the future.

## Conflicts of interest

None declared.

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## SUPPORTING DATA:

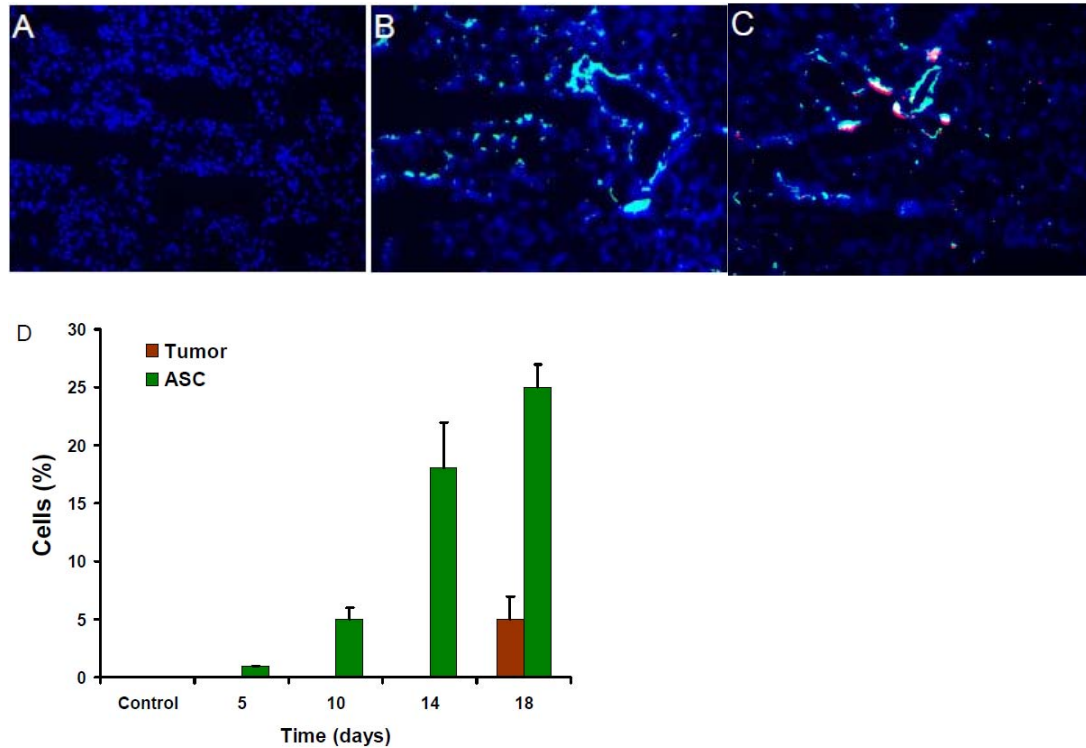


Figure 1. Adipose tissue derived stem cells form the premetastatic niche. **A**, GFP<sup>+</sup> ASCs are not found in the lungs after irradiation and before DsRed-tagged 4T1 cell implantation ( $n = 5$ ). **B**, On day 14, GFP<sup>+</sup> (green) ASCs are seen with no DsRed<sup>+</sup> (red) tumour cells ( $n = 5$ ). **C**, Beginning on day 18, a few single DsRed<sup>+</sup> 4T1 cancer cells adhere to GFP<sup>+</sup> ASC clusters ( $n=5$ ), **D**, A graph showing flow cytometric data of GFP<sup>+</sup> ASCs and DsRed<sup>+</sup> 4T1 cancer cells in the lung.

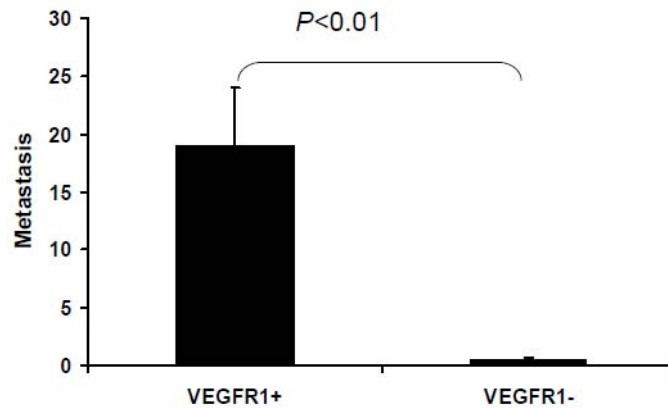


Figure 2. VEGFR1<sup>+</sup>-selected ASCs permits micrometastasis. VEGFR1<sup>+</sup> and VEGFR<sup>-</sup> cells were separated by cell sorting. Purity of VEGFR1<sup>+</sup> cells was 95%. The purified cells were injected intravenously ( $10^5$  cells) every three days for a total of 23 days. Animals were sacrificed 24 days after 4T1 tumor cell implantation. ASCs depleted of VEGFR1<sup>+</sup> cells abrogates both clusters and metastases (right panel) ( $P < 0.01$  by ANOVA). The graph shows the number of 4T1 micrometastases per x100 objective field.